Fascin can be an auxiliary immunomarker of ovarian granulosa cell tumors: comparison with calretinin and inhibin-α

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Summary

The histopathologic diagnosis of granulosa cell tumor adult type (AGCT) can be supported by the use of established immunomarkers such as inhibin-α and calretinin. Previously unreported data is presented on the detection of fascin in AGCT, in nonneoplastic granulosa cells and in other types of sex-cord stromal tumors. In addition, by staining a panel of various tumors, potentially included in the differential diagnosis of AGCT, we assessed the value of fascin as an auxiliary AGCT immunomarker. Intense and strong fascin staining may assist in cases with ambiguous calretinin or inhibin-α staining. On the contrary, absence of fascin should question a provisional morphologic diagnosis of AGCT.

Key words: Fascin; Ovary; Granulosa; Immunohistochemistry; Inhibin-α, calretinin.

Introduction

The histopathologic evaluation of adult granulosa cell tumors (AGCT) may become problematic since they are rare, present variable histologic patterns and can recur unpredictably [1, 2]. The application of immunohistochemical markers has assisted the morphologic assessment [3-11], but the available immunomarkers may show limitations regarding sensitivity or specificity.

Fascin-1 or simply fascin is a 55kDa actin-bundling protein [12-15]. Fascin cross-links actin filaments into tightly packed bundles, thus having a role in the formation of various actin-based cellular structures [16, 17]. Fascin is normally found in mesenchymal and neural tissues. Its expression is low or absent in non-neoplastic adult epithelial tissues, but may be increased in carcinomas [18]. Recent reports suggest that fascin may be a new prognostic indicator in several types of human carcinoma [19-29], probably due to its involvement in the formation of cellular surface protrusions and cellular motility. In vitro studies, based on transfection experiments, have shown that elevated levels of fascin increased the speed of cell migration and emphasized the association between fascin expression and motility of transformed cells [30].

In a previous study that examined fascin immunoreactivity in epithelial ovarian tumors [24] we observed strong staining in granulosa cells of ovarian follicles. The aim of the present study was to analyze fascin immunoreactivity in ovarian granulosa cell tumors and in a range of tumors that could potentially enter in their differential diagnosis. Thus, we could evaluate the possible role of fascin as a surrogate immunomarker in granulosa cell tumors.

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Materials and Methods

Patients and surgical specimens

Twenty-two ovarian granulosa cell tumors were included in the study, 21 adult-type (AGCTs) and one juvenile-type (JGCT). Nine of them were retrieved from the archives of the Pathology Department of the University Hospital of Larissa. The rest were seen in consultation and paraffin blocks were obtained from other hospitals in Greece. The age of the patients ranged from 23 to 67 years old. Nine of the patients showed various effects of hyperstrenism. Metastasis was histologically documented in only one case and multiple samples from the metastatic deposits were included in the study. The maximum dimension of the tumors ranged from 1-24 cm, whereas the median was 7 cm. The microscopic features of the tumors were conventional and represented most of the morphologic spectrum seen in AGCT.

The study also included 14 cases of ovarian sex-cord stromal tumors of other histological types and 101 cases of various neoplasms that could potentially enter in a differential diagnosis with AGCT. The sex-cord stromal tumors included four fibromas, one thecoma, three fibrothecomas, two Sertoli-Leydig cell tumors, two sclerosing stromal tumors and two unclassified sex-cord stromal tumors. In the group of various neoplasms we included 42 ovarian carcinomas, 15 breast lobular carcinomas, eight carcinoids (of lung, GI tract, ovary and uterine cervix), 14 small cell carcinomas (of lung or urinary bladder), six melanomas (three of them metastatic), seven mesotheliomas, two endometrioid stromal sarcomas and seven high-grade sarcomas. In each case one sample was included with the exception of lobular carcinomas where we added ten samples from metastatic sites. Additionally, we studied samples from six cases of peritoneal mesothelial hyperplasia.

Immunohistochemical procedures

We applied the following antibodies: for fascin (clone IM20, dilution 1:300, 20 min at room temperature [RT], Novocastra, Newcastle upon Tyne, U.K.), for inhibin-α (clone BC/R1, dilution 1:30, 20 min [RT], Biocare Medical, Walnut Creek, CA)

Table 1. — *Fascin immunoreactivity in sex cord-stromal tumors and miscellaneous tumors and lesions.*

Tumors or tumor-like lesions	Number of cases	IHS 0	IHS 1-3	IHS 4
Sex cord-stromal tumors				
Granulosa cell tumors, adult-type	21	0	0	21
Metastatic granulosa cell tumors	1	0	0	1[a]
Granulosa cell tumors, juvenile-type	1	0	0	1
Sertoli-Leydig cell tumors	2	0	0	2
Sclerosing stromal tumors	2	2	0	0
Fibromas/thecomas/fibrothecomas	8	0	1	7
Sex cord-stromal tumors, unclassified	2	0	1	1
Miscellaneous lesions				
Undifferentiated or poorly differentiated serous ovarian carcinomas	33	8	21	4
Endometrioid ovarian carcinomas	9	4	5	0
Endometrioid stromal sarcoma	2	0	1	1
Lobular carcinomas of the breast	15[b]	15	0	0
Carcinoids (lung, GI tract, ovary, uterine cervix)	8	8	0	0
Small cell carcinomas (lung, bladder)	14	1	10	3
Melanomas	6	5	1	0
Mesotheliomas	7	6	1	0
Sarcomas	7	3	2	2
Mesothelial cell hyperplasias	6	4	2	0

[a]In one slide of metastatic deposits of AGCT fascin expression was of moderate intensity in part of the slide.
[b]In three cases metastases to the lymph nodes (8) and one to the ovary and intestine were additionally examined (total number of 25 slides).

and for calretinin (polyclonal, dilution 1:50, 20 min [RT], Biocare Medical, Walnut Creek, CA). Immunohistochemistry was performed using a streptavidin-biotin-peroxidase method in a commercially available automated immunostainer (Bond Max, Vision Biosystems, Australia). For antigen retrieval Bond Epitope Retrieval Solution 2 (30 min, Vision BioSystems, Mount Waverley, Australia) was used for fascin and calretinin, while Bond Epitope Retrieval Solution 1 (20 min, Vision BioSystems, Mount Waverley, Australia) was used for inhibin-α. Binding of the primary antibodies was assessed by the Bond Polymer Refine Detection (Vision Biosystems, Newcastle upon Tyne, U.K.), with DAB as a chromogen. Antibodies for inhibin-α and calretinin were applied only to granulosa cell tumors.

Two aspects of fascin immunoreactivity were semiquantitatively evaluated, intensity and extent. Intensity was estimated by comparing tumor cell staining to that of adjacent endothelial cells, the latter being used as internal positive controls. Immunostaining was considered as "intense" when it was similar to that of endothelial cells (score 2), and "weak to moderate" (score 1) when it was less intense than that of endothelial cells. The extent of immunoreactivity was categorized according to the percentage of immunostained neoplastic cells into five groups with appropriate scores: negative (0), 1-25% (1+), 26%-50% (2+), 51%-75% (3+) and > 75% (4+). Using the same scoring system we estimated the tumor cell percentage showing an intensity score of 2. After preliminary analysis of the findings, the pathologists involved in the evaluation of the immunohistochemical staining realized that the visualized differences in the immunoreactivity among various cases were appreciated best by counting only the cellular subpopulation showing *"intense"* immunohistochemical staining, and expressing it as the highest immunohistochemical score (IHS). To calculate IHS a value was assigned for the percentage of the said subpopulation (0, 1: < 25%, 2 26-50%, 3: 51-75%, 4: > 75%), with IHS ranging from 0 to 4.

Inhibin-α and calretinin immunoreactivity was evaluated semiquantitatively according to the percentage of immunostained neoplastic cells and categorized into five groups: negative (0), 1-25% (1+), 26%-50% (2+), 51%-75% (3+) and >75% (4+) (8).

Western blotting

Western Blotting experiments were performed on proteins isolated from sections of tumor tissue kept at -80°C. Cells were lysed with NET-Triton Lysis Buffer (0.01 M Tris-Cl, 0.1 NaCl, 1 mM EDTA pH 7.4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate and a cocktail of protease inhibitors). Aliquots of lysates containing 10 μg of total protein for fascin detection were run on 8-12% NuPAGE Tris-Acetate gel (Invitrogen, Carlsbad, CA, USA) under denaturing and reducing conditions. Proteins were transferred to PVDF membranes (BioRad, USA). Nonspecific binding of antibody to the membrane was blocked by one-hour incubation with 5% (w/v) non-fat dry milk/0.01 (v/v) Tween 20 in PBS.

Immunoblot analysis was performed using mouse monoclonal anti-fascin (1:50 dilution, IM20, Novocastra, Newcastle upon Tyne, U.K.). Human β-actin monoclonal antibody (SIGMA, USA) was used as a protein marker for the quantification of the protein bands. Membranes were then immersed in ECL detection solution (Santa Cruz, USA) and exposed to XAR-5 film (Kodak, USA) for autoradiography. Protein bands were quantified using an Epson GT-8000 laser scanner. The ratios of fascin protein band intensity relative to β-actin band intensity were calculated for each sample.

Results

AGCTs showed extensive and "intense" staining (Figure 1). Notably, all cases showed IHS 4, the maximum score (Table 1). In most cases, fascin staining was weak or absent in the stroma surrounding nests and cords of AGCT. Thus, fascin immunostaining highlighted the epithelioid arrangement of AGCT, in a manner analogous to a reticulin stain (Figure 1). Fascin immunostaining was cytoplasmic (Figure 2). Two luteinized AGCTs showed more intense staining. Fascin immunoreactivity was not detected in theca cells adjacent to the granulosa cords and nests. These theca cells were immunostained with calretinin and inhibin-α (see below and Figure 7). In general, there was not any obvious difference in fascin staining between the various morphologic patterns of AGCT. However, rare foci with predominant spindle morphology or lack of epithelioid arrangements showed less intense staining (Figure 3). Also, one metastatic focus, in the single case available, showed slightly weaker fascin staining (Figure 4a).

The relative amount of fascin protein in granulosa cell tumor samples was also visualized by Western blotting analysis. In five of the cases included in the study tumor tissue kept at -80°C was tested. The median fascin/β-actin band intensity ratio was 0.63 (range 0.53-0.72), while for the positive and negative controls the ratio was 0.44 and 0.12, respectively. Representative examples are shown in Figure 8.

Fascin immunoreactivity was low in sclerosing stromal tumors (IHS 0, Figure 5). In Sertoli-Leydig cell tumors the Sertoli cell component showed intense staining, similar to that of AGCT. There was weaker staining in the Leydig cells and in the stroma. In two unclassified sex cord-stromal tumors the sex cord component showed fascin staining weaker than that of AGCT. In contrast, the stroma stained more intensely than that of AGCT and it was

Fig. 1

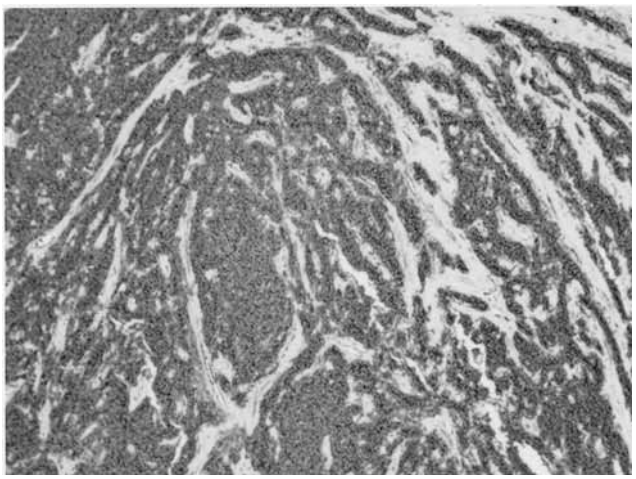


Fig. 2

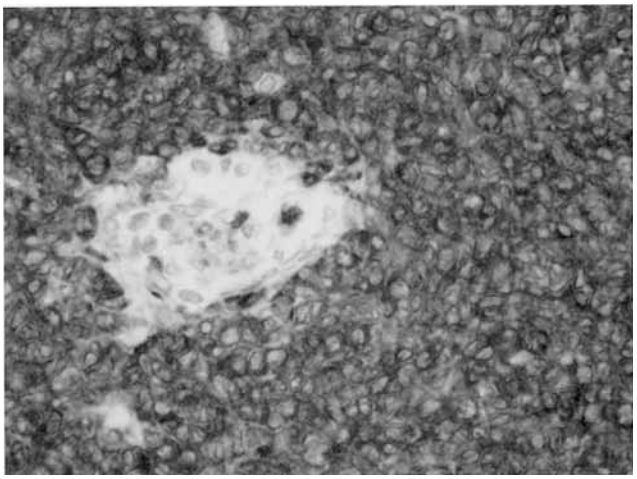


Fig. 3

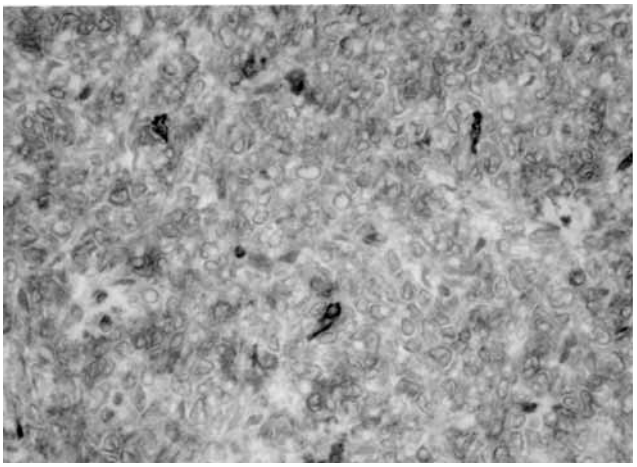


Fig. 4

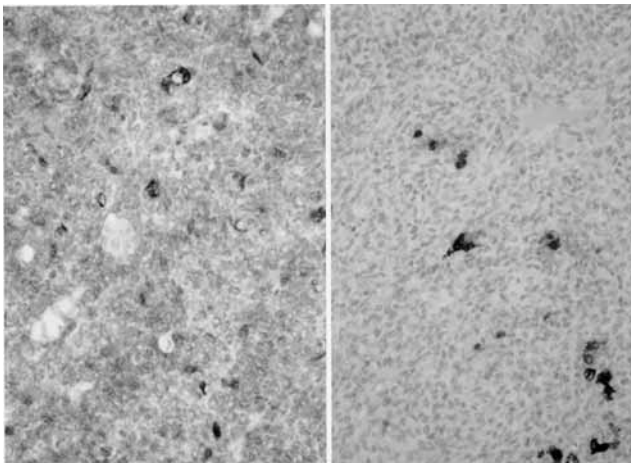


Fig. 5

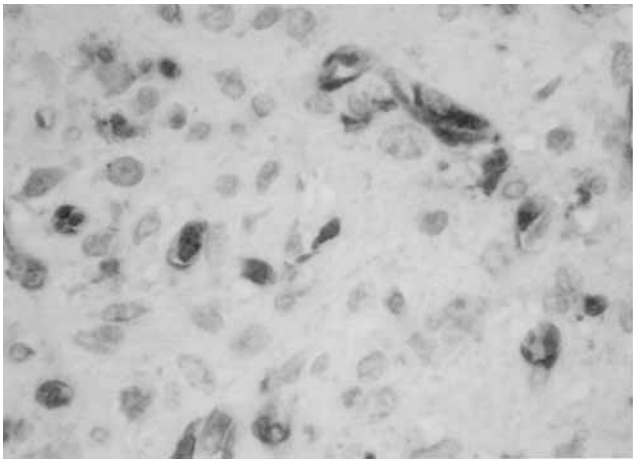


Fig. 6

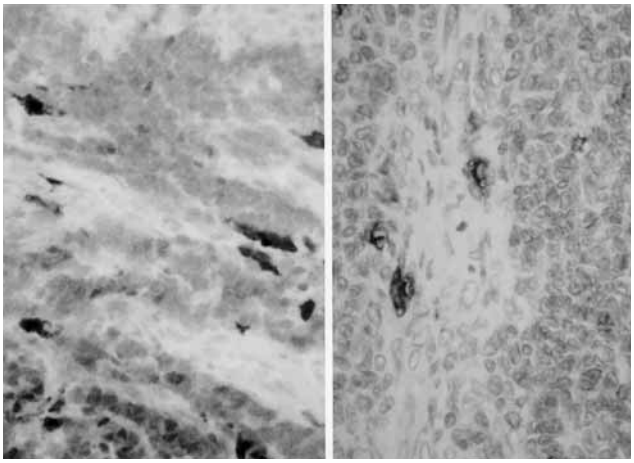


Figure 1. — AGCTs showing extensive and intense staining for fascin.

Figure 2. — Immunostaining for fascin was cytoplasmic.

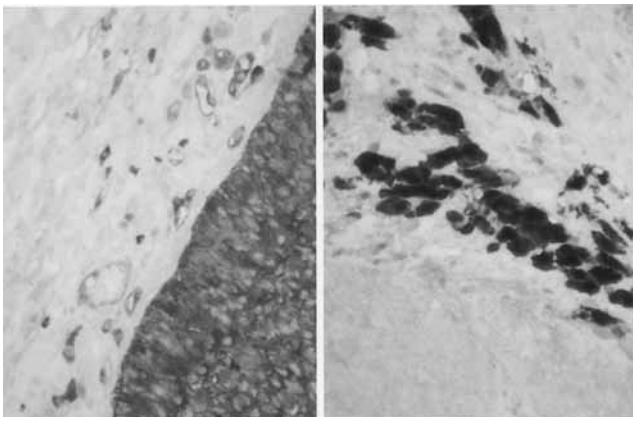
Figure 3. — Rare foci lacking obvious epithelioid arrangements showing less intense staining.

Figure 4. — One metastatic focus showing weaker fascin staining. Theca-like cells in metastasis were negative for fascin (a), but strongly positive for calretinin (b).

Figure 5. — Two sclerosing stromal tumors showing weak or absent immunoreactivity. Note positivity in endothelial cells.

Figure 6. — Immunostaining for calretinin (a) and inhibin-α (b) was often uneven or patchy.

Fig. 7



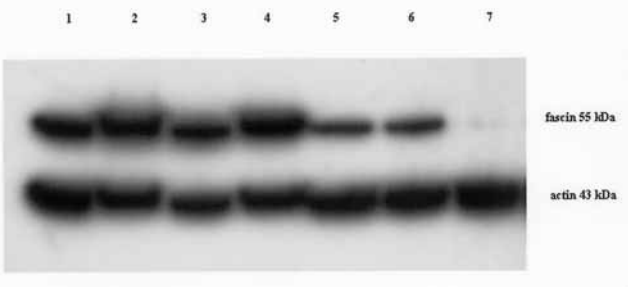


Fig. 8

Figure 7. — Fascin did not stain theca cells in the immediate vicinity of AGCT cords and nests (a), while calretinin (b) stained these cells strongly.
Figure 8. — Western blotting analysis of fascin protein in tumor samples and controls: Samples 1-4 represent AGCTs, samples 5-6 and 7 represent positive and negative controls, respectively.

similar to that of fibrothecomas. In fibrothecomatous tumors there was intense and extensive fascin staining.

Fascin immunostaining differed from that of calretinin and inhibin-α in several ways. Fascin stained more tumors and more cells in each tumor (Table 2). In addition, fascin staining was uniform whereas that of calretinin and inhibin-α was uneven or patchy (compare Figures 1 and 6a-b). Furthermore, fascin did not stain theca cells in the immediate vicinity of AGCT cords and nests (Figure 7a). Calretinin (Figure 7b) and inhibin-α stained these cells strongly.

Calretinin immunostaining was seen in 20 out of 21 AGCTs. Extensive calretinin staining (> 75% of tumor cells) was seen in 28.6% of the cases. Inhibin-α immunostaining was seen in 19 out of 21 AGCT. Extensive inhibin staining (> 75% of tumor cells) was seen in 14.3% of the cases. Immunoreactivity for calretinin and inhibin-α is summarized in Table 2.

Table 2. — *Immunoreactivity of AGCTs for calretinin and inhibin-α.*

Score	0	1+	2+	3+	4+	Total
Calretinin	1	3	5	6	6	21*
Inhibin-α	2	4	6	6	3	21*

*Number of cases.

From the 101 miscellaneous tumors and lesions, only ten showed fascin immunoreactivity comparable to that of AGCT, i.e., similar IHS. These included four ovarian carcinomas, three non-ovarian small cell carcinomas, two sarcomas and one endometrioid stromal sarcoma. Almost 50% of this group of cases showed no fascin staining at all.

Discussion

This is the first study that demonstrates the presence of fascin in AGCT and in non-neoplastic granulosa cells.

Detection of fascin in normal granulosa cells raises questions regarding fascin activity in these cells. Fascin could be related to microvillous processes observed in granulosa cells. The latter extend cytoplasmic projections, through zona pellucida, to connect with oocytes via gap junctions [31-33]. These connections are considered important for the proper preservation of the oocytes. Filopodia have been observed in AGCTs by electron microscopy [34]. In general, the formation of filopodia requires intensified actin assembly and in several cell types it is associated with overexpression of fascin [17]. Thus, fascin could be related to filopodia formation in granulosa cells.

Given the above comments, fascin immunoreactivity in neoplastic granulosa cells could be expected. Fascin is still expressed after neoplastic transformation as a preserved element of the granulosa cell phenotype. With the help of electron microscopy, astute observers have described interdigitating filopodia within Call-Exner bodies [35]. Since fascin could be a feature of well differentiated granulosa cell tumors, we looked in areas showing patterns considered to represent tumor with poorer differentiation. Overall, we did not observe a relationship of fascin immunostaining with the degree of differentiation in AGCT, although few areas showed slightly less fascin staining. Thus, fascin might not be a surrogate marker in grading AGCT.

The histopathologic diagnosis of AGCT depends primarily on the identification of traditional morphologic features. Immunohistochemistry plays an ancillary role. It may become critical in cases with poor preservation of morphology coupled with relative inexperience of the observer. The introduction of a new candidate immunomarker for AGCT should prompt comparison with two markers already applied routinely, inhibin-α and calretinin [3-11, 36]. Both of them appear to be more specific and less sensitive than fascin. However, fascin may still have a "role" in some diagnostic "scenarios". Absence of fascin immunoreactivity could help in excluding AGCT when the latter is low in a diagnostic list. On the contrary, finding uniform-strong fascin staining could reassure a pathologist issuing a diagnosis of AGCT without convincing immunoreactivity for inhibin-α or calretinin. AGCT-negative for these two markers was seen in 4.8% of our cases. Thus, the diagnostic contribution of fascin may be based on its negative predictive value.

The value of a new immunomarker may not always depend on its high diagnostic contribution. Occasionally, it may help in highlighting histogenetic points. In the case of AGCT, a neoplastic theca cell component has

been described and it has been noted in metastatic foci [37]. In our study, theca cells were stained with calretinin and inhibin but not fascin. In exceptional cases the above-mentioned theca cells were admixed with granulosa tumor cells. However, most of these cells were in the vicinity but outside the granulosa cell cords and nests. This pattern of localization could suggest that some theca cells were not genuinely neoplastic. However, we have noted the same cells in metastatic foci (Figure 7). Additional metastatic cases showing this pattern would further support the neoplastic nature of the theca cells.

Fascin appears to be a promising prognostic marker in some carcinomas. In those tumors it may be involved directly in the mechanisms of tumor cell migration. Theoretically, fascin could also be involved in granulosa cell metastasis, although we observed reduced fascin staining in metastatic foci. An improved general understanding of the metastatic mechanisms involving fascin could offer a new therapeutic target in AGCT management in the future.

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